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Research paper

Overexpression of Lewis y antigen promotes human epididymis protein 4-mediated invasion and metastasis of ovarian cancer cells

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ABSTRACT

To study Human epididymis protein 4 (HE4) surface fucosylation and to determine the effects and significance of Lewis y antigen on HE4-mediated invasion and metastasis of ovarian cancer cells, we investigated four types of ovarian cancer cells and found that six fucosylated antigens (Lewis y, Lewis x, Lewis a, Lewis b, sLewis a, and sLewis x) were identified on HE4 in ovarian cancer cells. Moreover, modification of the type II sugar chain (Lewis y, Lewis x, and sLewis x) was significantly higher than the type I sugar chain (Lewis a, Lewis b, sLewis a) of the lactose series. To confirm the effects of Lewis y antigen on HE4-mediated invasion and metastasis of ovarian cancer cells, the CaoV-3 cells with high Lewis y antigen on the HE4 surface and ES-2 cells, with high Lewis x antigen but low Lewis y antigen, were investigated. We found that the expression levels of HE4 and Lewis y increased in both cell lines while the level of Lewis x didn't have any change after transfection. Furthermore, the high expression of Lewis y antigen significantly enhanced the HE4-mediated invasion and metastasis of ovarian cancer cells. The invasion and metastasis capacities were significantly decreased after Lewis y antibody blocking. This study demonstrates that overexpression of the Lewis y antigen on HE4 promotes ovarian cancer cell invasion and metastasis, which is likely to be used as a target for the clinical treatment of ovarian cancer.

1. Introduction

Ovarian cancer currently has the highest mortality of reproductive system tumors globally. Due to a lack of effective treatment, the five-year survival of ovarian cancer patients is less than 50% [1]. However, patients diagnosed early, and properly treated, obtain a significantly higher 5-year survival rate (up to 90%). Therefore, the exploration for early diagnostic biomarkers of ovarian cancer has become a research hotspot [2].

Human epididymis protein 4 (HE4), also known as whey acidic protein (WFDC2), was first found in the epididymis [3] and has been designated by the FDA as a serum marker of ovarian cancer [4]. Recent studies have shown that HE4 is not only highly expressed in ovarian cancer but is also relatively highly expressed in endometrial cancer, lung cancer, and other malignant tumors [5,6].

In 2005, HE4 was recognized as a secretory glycoprotein [7]. Glycosylated antigens widely expressed on the cell membrane are important components of glycoproteins. Their changes are closely related with biological characteristics of malignant cells such as proliferation, invasion, and metastasis. Fucose is widely involved in the composition of sugar chains and changes in the sugar chain structures of fucose are related with the malignancy of some cancer cells [8]. Lewis glycans, fucosylated glycans of the lactose series, have also been shown to be important. Lewis glycans include Lewis y, Lewis x, Lewis a, Lewis b, sialyl Lewis a (sLewis a), and sLewis x with type I (or type II) sugar chains. Similar to sialic acid, it is generally presumed that once the terminal residues of the sugar chain are connected to a fucosyl group, sugar chain synthesis will stop.

Our previous studies showed that Lewis y antigens are expressed on HE4 in ovarian cancer cells as well as in tissues [9]. However, it is currently unclear whether other fucosylation occurs on the HE4 surface and which type of Lewis antigens expressed on HE4 play an important role in the malignant behavior of cancer cells, particularly ovarian cancer cells.

Abbreviations: HE4, human epididymis protein 4; sLewis a, sialyl Lewis a.

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In this study, the expression levels of fucosylated antigens on HE4 in ovarian cancer cells were detected, demonstrating the prevalence of fucosylation on HE4. Our results also found that the expression levels of type II sugar chains were significantly higher than type I sugar chains on HE4 in ovarian cancer cells. Moreover, HE4 significantly enhanced the invasive and metastatic capacities of ovarian cancer cells and this was related to the high expression of Lewis y antigen on HE4.

2. Materials and methods

2.1. Cell lines and cell culture

OVCAR-3, SKOV-3, ES-2 and CaoV-3 ovarian cancer cell lines were purchased from Cell Culture Collection of ShangHai. OVCAR-3, SKOV-3, ES-2 were propagated in McCoy's 5 A (Invitrogen) modified medium and CaoV-3 was propagated in 1640 (Hyclone) modified medium with 10% fetal bovine serum.

2.2. Cell transfection and establishment of stable-transfected cell lines

Full-length coding sequences of HE4 were cloned using PCR, and the expression vector pcDNA3.1-HE4-H was constructed. The primer sequences were: P1: 5′-TCC GCT CGA GAT GCC TGC TTG TCG CCT AG-3′ and P2: 5′-ATG GGG TAC CGT GAA ATT GGG AGT GAC ACA GG-3′. The expression vectors and empty vectors were transfected into the ovarian cancer cell lines ES-2 and CaoV-3 by lipofection 2000 (Invitrogen). G418 (800 μg/ml, Invitrogen) was used in the screening of cells to establish cell lines with stable overexpression of the HE4 gene as well as the corresponding empty vectors, which were named as ES-2/pCD-HE4, ES-2/pCD-mock (ES-2 stable cells) and CaoV-3/pCD-HE4, CaoV-3/pCD-mock (CaoV-3 stable cells), respectively.

2.3. Immunocytochemistry

Cells at exponential phase of growth were digested by 0.25% trypsin and cultured in medium containing 10% FBS to prepare single-cell suspension. Cells were washed twice with cold PBS when growing in a single layer, and fixed with 4% para- formaldehyde for 30 min. The expression of HE4 and Lewis y on cells were detected according to the SABC kit instructions. The concentration of HE4 monoclonal (Abcam, Rabbit) and Lewis y monoclonal (Abcam, Mouse) antibody were 1:300 and 1:100, separately. The primary antibody was replaced by rabbit IgG (Bioss, China) and mouse IgM (Bioss, China) for negative control.

2.4. Real-time PCR

Cells were added with Trizol reagent (1 mL per 1×10^7 cells) to extract total RNA. cDNA was synthesized according to the RNA reverse transcription kit instructions (TAKALA, Da Lian, DRR047A). The sequences of HE4 gene primers were 5′-AGT GTC CTG GCC AGA TGA AAT G-3′ for forward primer and 5′-CAG GTG GGC TGG AAC CAG AT-3′ for reverse primer. The sequences of FUT1 gene primers were 5′-TGG TTG GGA AAG GGA GAA-3′ for forward primer and 5′-CGC CTG CTT CAC CAC CTT CTT G-3′ for reverse primer. The sequences of GAPDH primers were 5′-ATG GAA ATC CCA TCA CCA TCT T-3′ for forward primer and 5′-CGC CCC ACT TGA TTT TGG-3′ for reverse primer. The Light Cycler PCR system (Roche Diagnostics, Mannheim, Germany) was used for real-time PCR amplification and Ct value detection. The melting curves were analyzed after amplification. PCR reactions of each sample were done in triplicate. Data were analyzed through the comparative threshold cycle (CT) method.

2.5. Co-immunoprecipitation

Ice-cold RIPA buffer (1 ml) was added to ovarian cancer cells, followed by incubation at 4 °C for 30 min. After centrifugation at 15,000 × g for 30 min at 4 °C, supernatant fractions were collected and treated with anti-HE4 antibody (10 μl) (Santa Cruz, goat polyclone) for 3 h at 4 °C. Protein A/G PLUS-Agarose (20 μl; Santa Cruz) was added, followed by incubation on a rocker platform overnight at 4 °C. The negative control contained only 10 μl HE4 antibody (Santa Cruz, goat polyclonal) without protein. The procedure was followed as described previously [9]. Immunoprecipitates were subsequently subjected to 12% SDS gel electrophoresis and analyzed via Western blot using HE4 monoclonal (Abcam, Rabbit), Lewis y monoclonal (Abcam, Mouse), Lewis x polyclonal (Santa Cruz, Mouse), sialyl Lewis x monoclonal (Lifespan, Mouse), Lewis a monoclonal (Abcam, Mouse), Lewis b monoclonal (Abcam, Mouse) and sialyl Lewis a monoclonal (Millipore, Mouse) antibodies. Proteins were visualized using ECL reagent (Amersham ECL Prime detection). Experiments were repeated three times.

2.6. Sandwich ELISA

Ninety- six-well polystyrene microplates were coated with a capture antibody against HE4 (Santa Cruz, goat polyclone) at 5 μg/ml in coating buffer at 4 °C for 16 h. After blocking with 5% BSA, 100 μl of the cell supernatants were added to the wells and incubated at room temperature for 2 h. Then, the plates were incubated with Lewis y, Lewis x, sLewis x, Lewis a, Lewis b and sLewis a mAbs followed by peroxidase-labeled goat anti-mouse IgM or IgG antibody. Color reaction was developed with o-phenylenediamine dihydrochloride solution at room temperature for 20 min. The reaction was stopped with 2.5 M sulfuric acid. Negative controls were performed with 1% BSA instead of the mAbs. The optical density of each well was determined within 30 min using a microplate reader at 450 nm [10].

2.7. Double-labeling immunofluorescence method

OVCAR-3 cells at exponential phase of growth were digested by 0.25% trypsin and cultured in medium containing 10% FBS to prepare single-cell suspension. Cells were washed twice with cold PBS when growing in a single layer, and fixed with 4% para-formaldehyde for 30 min. The cells were simultaneously incubated with primary antibodies against HE4 (1:100) and Lewis y (1:100). The primary antibody was replaced by rabbit IgG (Bioss, China) and mouse IgM (Bioss, China) for negative control. The working concentrations of fluorescein isothiocyanate and TRITC were 1:100. Nuclei were counterstained with DAPI. The empirical procedure was performed according to the manufacturer's instructions.

2.8. Wound healing, transwell assays and antibody blocking tests

Wound healing assay: Cells during the log phase were selected and single cell suspensions were prepared. Cells on a 6 well plate were cultured until 90% density. And then scrathed the plate straightly with 200 ul pipette. Cells were cultured in medium without serum. After 24 h, the width of the scarification were observed under microscope. Transwell assay: The Matrigel were melted and put at 4 °C refrigerator overnight the day before this experiment. The pipette tip was pre-cooled in ice-cold for 0.5 h during experiment, and the ECM gel was diluted by 1:8 with serum free medium, Matrigel 100 ul was added into the upper chambers,

the whole process was performed on ice. Then they were placed in an incubator at 37 °C for 5 h. 10^5/mL cells in logarithmic growth phase was added in each well for 200 ul, 500 ul medium supplemented with 10% fetal bovine serum were added in lower chamber. After culturing for 24 h, nutrient solution was abandoned and a cotton swab was used to gently wipe out the upper layer of transwell. Membrane of transwell was fixed with methanol for 20 min, washed with PBS 3 times, then staining with 0.1% crystal violet for 20 min after airing. The invasive cell numbers of 5 fields (upper and lower, left and right, middle) were counted under microscope, the mean value was obtained and the statistical analysis was made.

Cells during the log phase were selected and single cell suspensions were prepared. Lewis y mAb (10 μg/ml) was added to the adherent cells. Mouse IgM isotype control and PBS blank control groups were set up. The cells were left to stand at 37 °C for 30 min. The experiments were repeated three times and the average value was taken. In transwell assays, Lewis y mAb (10 μg/ml) was added into the upper chamber while paving the cells. The cells of each group were treated in triplicate and experiments were repeated three times.

2.9. Statistical analysis

SPSS version 17.0 (SPSS Inc, Chicago, IL) software was used for statistical analysis. $\chi 2$ analysis, variance analysis, and *t*-test were employed. $P < 0.05$ was considered statistically significant. Graph

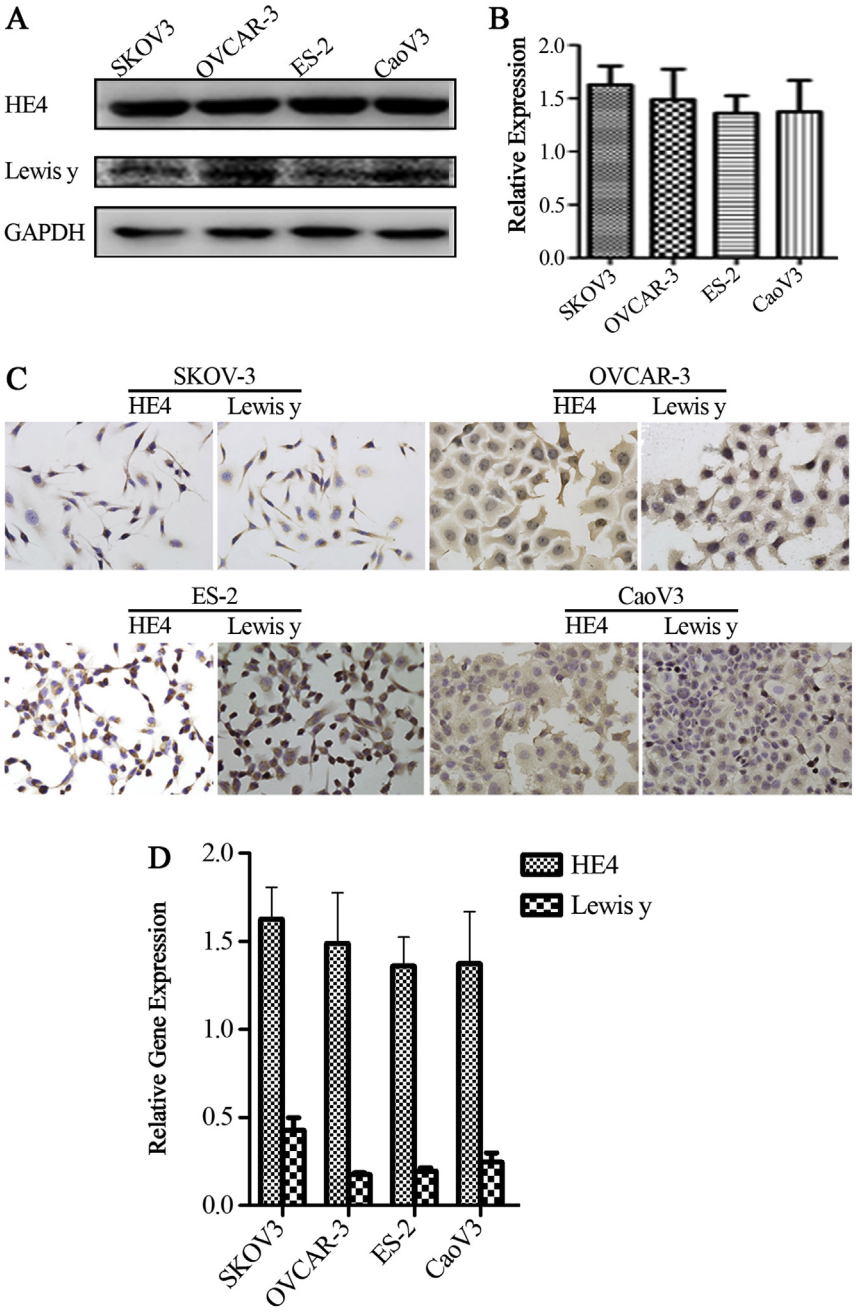

Fig. 1. The expression of HE4 and Lewis y antigen in ovarian cancer cells. The immunoblot shows the difference in HE4 and Lewis y expression among the 4 types of cancer cells (A). Quantitative data in panel B are expressed as HE4 to GAPDH. Immunocytochemistry (C, original magnification × 400) and Real-time PCR (D) show the expression of HE4 and Lewis y among these cancer cells, respectively.

Pad 5.0. Image J software was used to analysis westernblot semiquantitatively.

3. Results

3.1. The expression of HE4 and Lewis y antigen in ovarian cancer cells

We first investigated four types of ovarian cancer cells. HE4 and Lewis y expression levels in these four cells were detected using Western blot, immunocytochemistry, and real-time PCR assays. The results showed that HE4 and Lewis y antigens were expressed in all four cells (Fig. 1). The Western blot assays (Fig. 1A and B), immunocytochemistry assays (Fig. 1C) and real-time PCR assays (Fig. 1D) gave consistent results. Ovarian cancer cells all had relatively high HE4 expression levels. However, the Lewis y expression levels in these four cells were inconsistent, which might be related to the fact that Lewis y is present on the surface of various tumor-associated proteins and not just HE4. Immunocytochemistry assays found that the HE4 proteins were stained as brown or light yellow granules distributed in the cytoplasm and were also in the membrane and around the nuclei. Lewis y antigens also stained positive and were widely distributed in the membrane and cytoplasm (Fig. 1C).

3.2. Co-expression of HE4 and Lewis antigens in ovarian cancer cells

Our previous investigations confirmed that Lewis y antigens are present on HE4. To further investigate whether there are other fucosylations on the HE4 surface, co-immunoprecipitation, sandwich ELISA, and double-labeling immunofluorescence assays were applied in the above four types of cancer cells. The results of co-immunoprecipitation suggested that Lewis y, Lewis x, sLewis x, Lewis a, Lewis b, and sLewis a fucosylations were expressed on the HE4 surface in all ovarian cancer cells (Fig. 2A). However, the expression levels of type II sugar chains (Lewis y, Lewis x, and sLewis x) were significantly higher than type I sugar chains (Lewis a, Lewis b and sLewis a) in these cells ($P < 0.05$). The results from the sandwich ELISA assay were largely consistent with these results (Table 1). The double-labeling immunofluorescence assay also detected the colocalization of HE4 and Lewis antigens in all these cells, and the colocalization of HE4 and Lewis y antigen were most evident in ovarian cancer cell lines. HE4 was mostly located in the cell membrane and cytoplasm of the ovarian cancer cell line OVCAR3 (Fig. 2B). Lewis y antigen was also mainly located in the cell membrane and cytoplasm. Two-channel composite channels confirmed the colocalization of HE4 and Lewis y antigen.

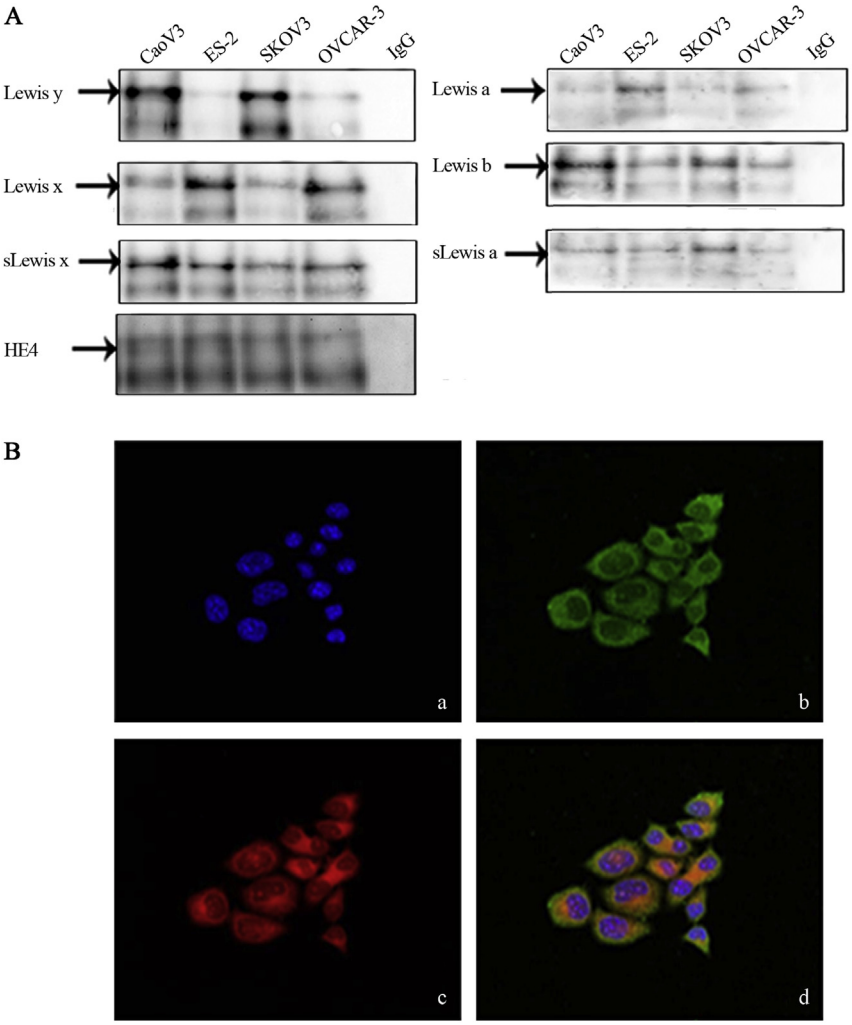

Fig. 2. Co-expression of HE4 and Lewis antigens in ovarian cancer cells. The immunoprecipitation shows the Lewis y, Lewis x, sLewis x, Lewis a, Lewis b, and sLewis a fucosylations were expressed on the HE4 surface in all four ovarian cancer cells (A). All the arrows indicate molecular weight (25 kDa). The double-labeling immunofluorescence shows the colocalization of HE4 and Lewis y antigen in ovarian cancer cell line OVCAR3 (B, original magnification × 400). Nucleus (a), Lewis y (b), HE4 (c) and merged image (d).

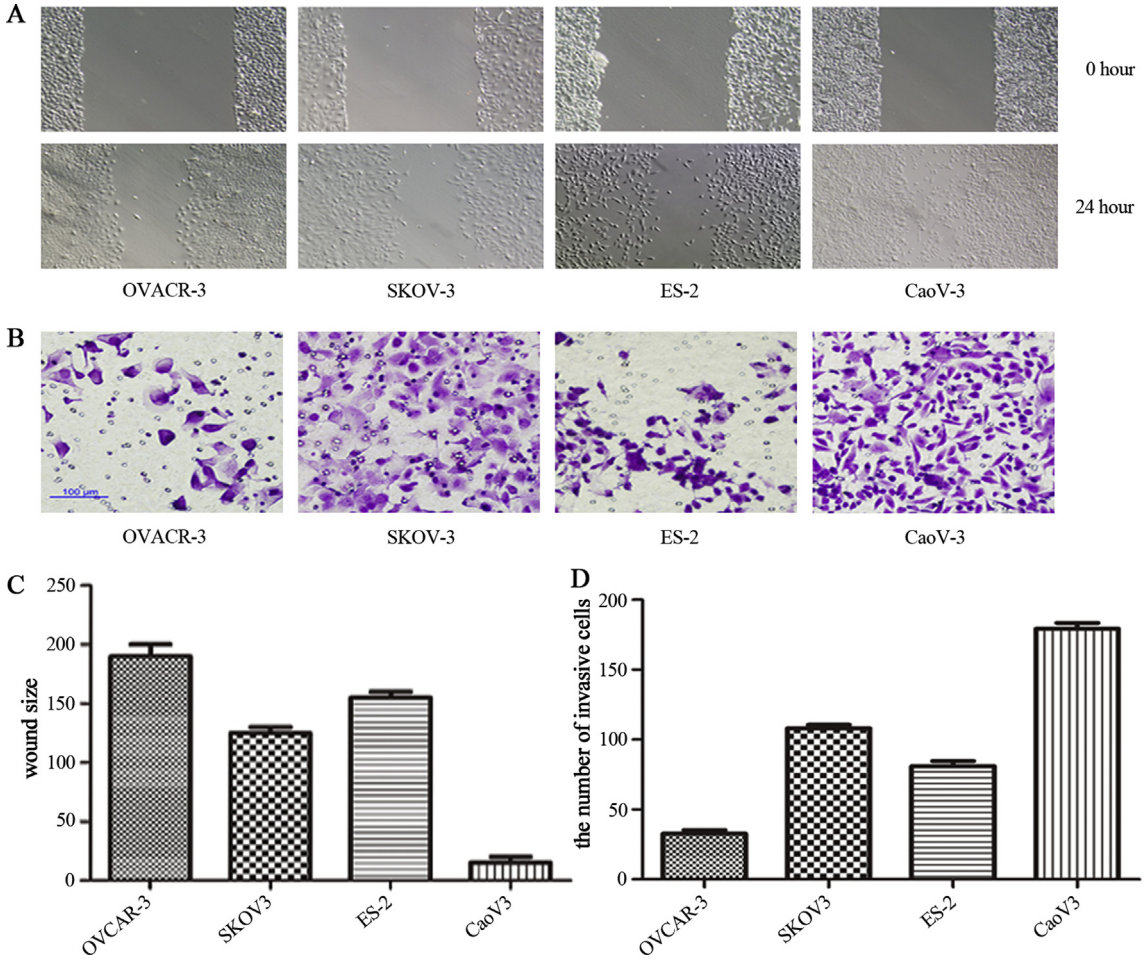

Fig. 3. The invasive and metastatic capacities of four types of ovarian cancer cells. Wound healing assay indicates the metastatic capacities of ovarian cancer cells (A, original magnification × 40). The metastatic capacities of the ovarian cancer cell lines ES-2 and OVCAR-3 were lower than CaoV-3 and SKOV-3 ($P < 0.01$, panel C). Transwell assay indicates the invasive capacities of ovarian cancer cells (B, original magnification × 200) and the invasive capacities of the ovarian cancer cell lines CaoV-3 and SKOV-3 were significantly higher than ES-2 and OVCAR-3 ($P < 0.01$, panel D).

3.3. The overexpression of Lewis y on HE4 promotes ovarian cancer cell invasion and metastasis

To investigate the effect of HE4 on ovarian cancer cell invasion and metastasis, wound healing and transwell assays were performed in four types of ovarian cancer cells. The metastatic and invasive capacities of the ovarian cancer cell lines ES-2 and OVCAR-3 were lower than CaoV-3 and SKOV-3 ($P < 0.01$, Fig. 3). On the basis of co-immunoprecipitation and sandwich ELISA results, the relative expression of Lewis y antigen on the HE4 surface in CaoV-3 and SKOV-3 cells was higher than its expression on ES-2 and OVCAR-3 cells (Fig. 2A, Table 1). In contrast, the relative expression of Lewis x antigen on the HE4 surface in CaoV-3 and SKOV-3 cells was lower than its expression on ES-2 and OVCAR-3 cells (Fig. 2A, Table 1).

Table 1
Binding of six kinds of Lewis antibodies with HE4 as catcher antibody in ovarian cancer cells. Score according to OD450 values minus blank as follows: ++, >0.5; +, 0.1–0.5; (+), 0.05–0.1; –, <0.05.

Cell lines	OVCAR-3	SKOV-3	ES-2	CaoV-3
$HE4^+$/Lewis y^+	+	++	+	++
$HE4^+$/Lewis x^+	++	+	++	+
$HE4^+$/sLewis x^+	+	+	++	+
$HE4^+$/Lewis a^+	–	–	(+)	–
$HE4^+$/Lewis b^+	(+)	+	(+)	+
$HE4^+$/sLewis a^+	–	(+)	–	–

To confirm the effects of the Lewis y antigen on the HE4-mediated invasion and metastasis of the ovarian cancer cells, CaoV-3 cells with high Lewis y antigen expression on HE4 and ES-2 cells (cells with high Lewis x antigen expression but low Lewis y antigen expression) were selected. ES-2 and CaoV-3 cell lines stably transfected with HE4 were constructed. After transfection, the expression levels of HE4 and Lewis y increased in both cell lines while the level of Lewis x didn't have any change ($P < 0.01$, Fig. 4). The invasive and metastatic capacities of the two types of cells were also enhanced, especially in ES-2 cells ($P < 0.01$, Figs. 5 and 6). We also found that the metastatic and invasion capacities of the two types of cells changed after dealing with Lewis y antibody. Compared with the control group, the metastatic capacity significantly weakened at 12 h and the invasion capacity significantly weakened at 24 h in the Lewis y antibody-blocked group ($P < 0.01$, Fig. 6). This result showed that the overexpression of Lewis y on HE4 significantly promotes ovarian cancer cell invasion and metastasis.

4. Discussion

The early metastasis and invasion of ovarian cancer cells are important factors related to a high death rate and prognosis.

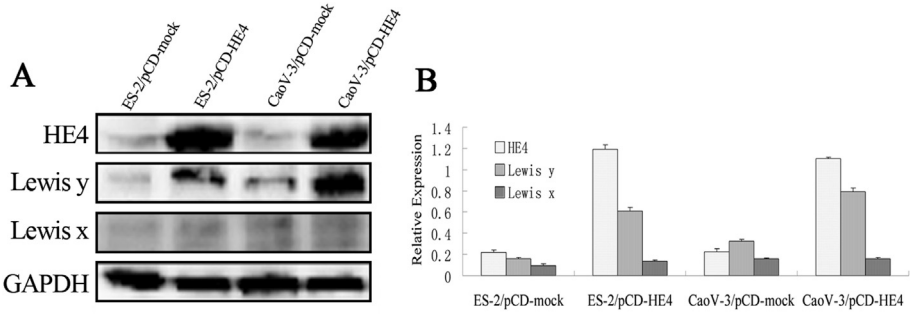

Fig. 4. The expression levels of HE4, Lewis y and Lewis x in ovarian cancer cells before and after HE4 gene transfection. The immunoblot shows the expression levels of HE4, Lewis y and Lewis x increased in both cell lines after transfection (A). Quantitative data in panel B are expressed as HE4 or Lewis y to GAPDH.

Invasion and metastasis are complex pathophysiological processes that not only involve interactions of cancer cells and host cells but also complex mutual regulatory mechanisms between many molecules with biological activities. Recent studies [11,12] have found that the invasion and metastasis of malignant ovarian cancer cells are significantly enhanced with HE4 overexpression. The invasive and metastatic capacities were weakened after the silencing of HE4, but the exact mechanisms are unknown. Our results confirm the important role of HE4; furthermore, we also found that the fucosylation of HE4 influences its invasive and metastatic capacities. Lewis y expression on the HE4 surface especially had a significant impact on invasion and metastasis.

The Lewis y antigen is an oligosaccharide containing a double fucose. Different levels of Lewis y overexpression have been found to occur in 75% of epithelial ovarian cancer cells, and patients with increased expression of the Lewis y antigen had a poor prognosis [13–15]. In addition to the CA125 and MUC-1 tumor markers of epithelial ovarian cancer [16], Lewis y antigens have been found on the surface of the epidermal growth factor receptor, integrin $\alpha_5\beta_1$, and CD44 in our previous study [17–19]. Lewis y antigen was also present on the newly discovered epithelial ovarian cancer tumor marker HE4 [9]. Our previous studies showed that Lewis y antigens expressed by cancer cells are involved in many cellular functions that influence the malignant behaviors of the cells, including adhesion, recognition, and signal transduction, by changing the phosphorylation levels of receptor signaling pathways. The increase of Lewis y antigens has been shown to be conducive to cancer cell invasion and spread [20].

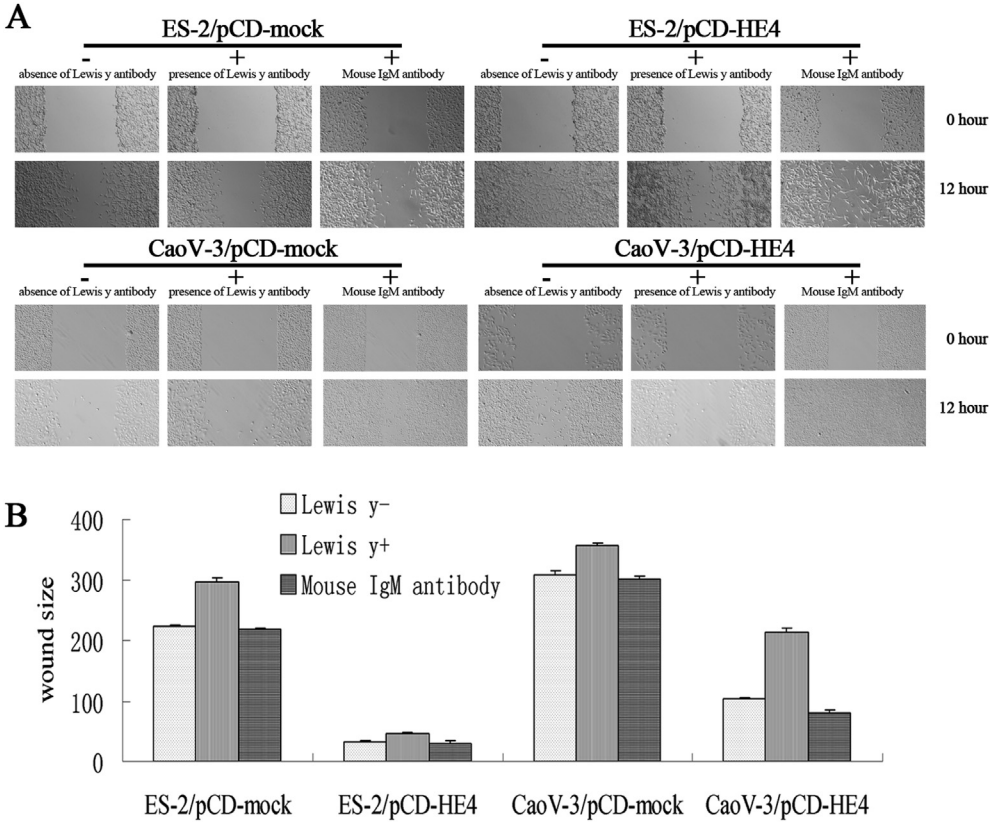

Fig. 5. The metastatic capacities of ovarian cancer cells before and after HE4 gene transfection. The metastatic (A, original magnification × 40) capacities of ovarian cancer cells before and after HE4 gene transfection. Plus sign means the Lewis y antibody-blocked groups while minus sign means the groups without Lewis y antibody. Mouse IgM antibody represents the negative control. Compared with the control group, the metastatic capacity (B) significantly weakened at 12 h in the Lewis y antibody-blocked group.

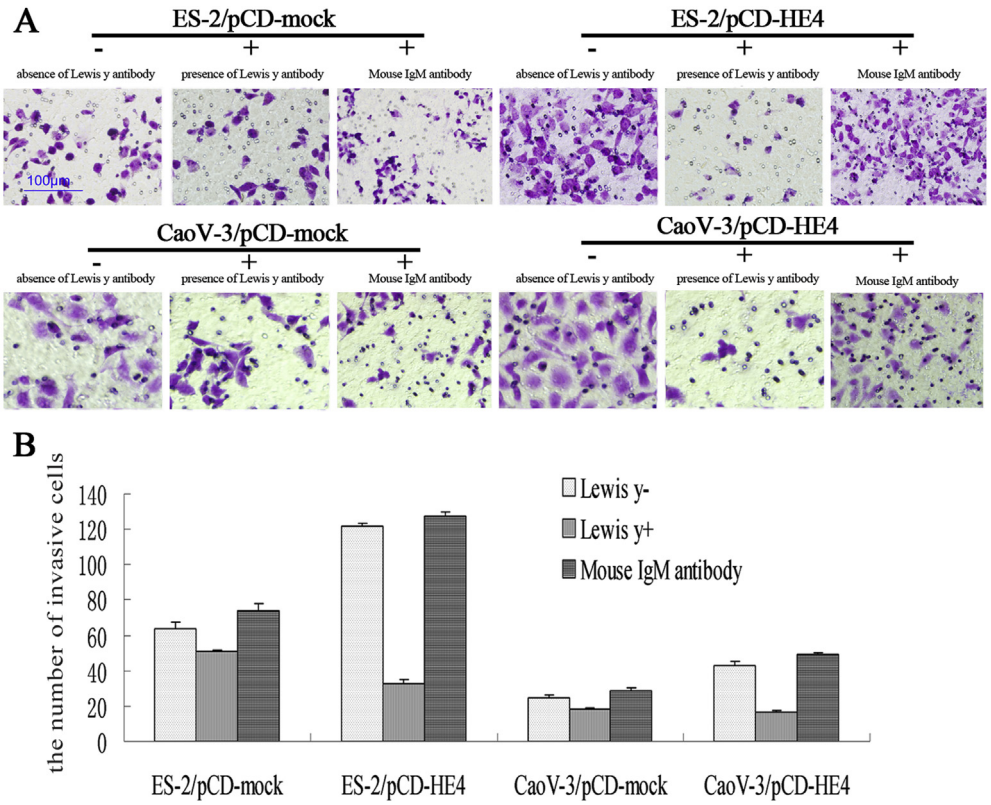

Fig. 6. The invasive capacities of ovarian cancer cells before and after HE4 gene transfection. The invasive (A, original magnification × 200) capacities of ovarian cancer cells before and after HE4 gene transfection. Plus sign means the Lewis y antibody-blocked groups while minus sign means the groups without Lewis y antibody. Mouse IgM antibody represents the negative control. Compared with the control group, the invasion capacity (B) significantly weakened at 24 h in the Lewis y antibody-blocked group.

The present study revealed that the fucosylation of HE4 is present in ovarian cancer cells. This study also demonstrated that besides the Lewis y antigen, modifications by Lewis x, Lewis a, Lewis b, sLewis, and sLewis x antigens were detected on the HE4 surface. The levels of such modifications were different in various cell lines. However, modifications with the lactose series type II sugar chains (Lewis y, Lewis x, and sLewis x) were significantly higher modifications with the type I sugar chain (Lewis a, Lewis b and sLewis a) in ovarian cancer cell, which might be associated with the high expression of alpha (1, 2) fucosyltransferase gene in the cell lines [21].

The metastatic and invasive capacities of the ovarian cancer cell lines CaoV-3 and SKOV-3 were higher than ES-2 and OVCAR-3 ($P < 0.01$). Meanwhile, the results of co-immunoprecipitation and sandwich ELISA assays proved that the relative expression levels of Lewis y antigens on the HE4 surface in CaoV-3 and SKOV-3 cells were also higher than expression levels in ES-2 and OVCAR-3 cells, while the relative expression levels of Lewis x antigens in CaoV-3 and SKOV-3 were lower than expression levels in ES-2 and OVCAR-3. These findings suggest that the Lewis x antigens in cells with higher metastatic and invasive capacities continue to be glycosylated, and that most are present as Lewis y antigens.

The Lewis y antigen contains a double fucose and has higher substrate adhesiveness compared with Lewis x [18,21]. To confirm the effects of Lewis y antigen on HE4-mediated invasion and metastasis of ovarian cancer cells, the CaoV-3 cells with high Lewis y antigen on the HE4 surface and ES-2 cells, with high Lewis x antigen but low Lewis y antigen, were investigated. After HE4 transfection, the expression levels of HE4, Lewis y, and Lewis y on the HE4 surface increased in the two types of cells. Furthermore, the invasive and metastatic capacities of the two types of cells after transfection were enhanced compared with the cells before transfection, especially in ES-2 cells. Lewis y antibody blocking experiments proved that the metastatic capacity weakened by 6 h after transfection, and the invasive capacity significantly weakened by 24 h after transfection. Furthermore, the migration and invasive capacities of the cells in the antibody-blocked group after transfection were significantly lower. These results therefore show that Lewis y overexpression on the HE4 surface significantly promotes ovarian cancer cell invasion and metastasis.

In this experiment we confirmed that Lewis y antigen overexpression on the HE4 surface promotes ovarian cancer cell invasion and metastasis. The invasive and metastatic capacities significantly decreased Lewis y antibody blocking. This still leaves the questions as to whether there are other associated proteins or pathways involved in Lewis y-mediated invasion and metastasis of ovarian cancer cells. In addition, whether Lewis y antigen overexpression on the HE4 surface affects proliferation, drug resistance, autophagy, and other biological behaviors of the ovarian cancer cells remains to be investigated.

A variety of anti-cancer drugs targeting Lewis y have been successfully developed in recent years [22–24], which opens up broad prospects for the treatment of malignant tumors. As HE4 is an FDA-approved tumor marker for monitoring the recurrence and progression of epithelial ovarian cancer [4], it is worthwhile to investigate whether the overexpression of Lewis y antigen on the HE4 surface can be used as a target for the clinical treatment of ovarian cancer.

5. Conclusion

The high expression of Lewis y antigen significantly enhanced the HE4-mediated invasion and metastasis of ovarian cancer cells.

The invasion and metastasis capacities were significantly decreased after Lewis y antibody blocking.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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